THE JEREMIAH METZGER LECTURE

FROM POMC TO FUNCTIONAL DIVERSITY OF NEURAL PEPTIDES: THE KEY IMPORTANCE OF CONVERTASES*,**

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MONTRÉAL

In 1979 (1) we published a review article which summarized the evolution of a biosynthetic model proposed in 1967. On the basis of the amino acid sequences of a series of pituitary peptides, we had suggested (2) that β- and γ-lipotropin (LPH) and β-melanotropin (MSH) could be part of a cascade of post-translational cleavages at pairs of basic amino acid residues (Figure 1). The discovery of β-endorphin (END) in 1976 (3) gave our model some flying colors since β-END is the C-terminal fragment of β-LPH.

Several series of experiments started by other groups in the early 1970's on the molecular forms of adrenocorticotropin (ACTH) (4) led to the possibility that ACTH is also synthesized as a precursor form (5). By means of elegant experiments on ACTH biosynthesis (6) and on its mRNA translation aspects (7), Herbert et al. in Oregon confirmed this hypothesis. Moreover, they found that ACTH and β-LPH originate from the same precursor. Thus, a large protein contains different end products (β-MSH, α-MSH, ACTH, β-END) having different biological activities. In 1979, we proposed (1) that this precursor be named pro-opiomelanocortin (POMC) to describe the known biological activities of its fragments (opioid, melanocyte stimulating and adrenocorticotropic).

The fact that β-endorphin has potent opioid activity (8), that Guillemin had found it in the hypothalamus (9) and that Krieger (10) had shown the presence of other POMC fragments in the brain brought the POMC

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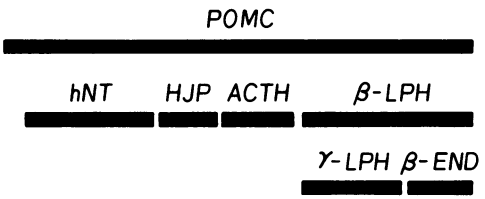

FIG. 1. The structure of pro-opiomelanocortin: beta- and gamma-LPH are parts of a cascade of post-translational cleavages.

model into the field of neuropeptides. Soon after, several groups demonstrated that many other neuropeptides are fabricated from large precursors (11, 12). This has now been confirmed for all brain neuropeptides (13). I have been fortunate enough to participate in this exciting field since my post-doctoral years in the laboratory of C.H.Li in Berkeley in the mid 1960's.

In this introduction we describe some of our current thoughts on the subject of peptide biosynthesis. Determination of the whole precursor molecule sequence allowed the discovery of numerous potentially active new peptide substances. One of the earliest examples was the N-terminal fragment of POMC (N-POMC) which has been isolated and sequenced in our laboratory: the search for its possible biological function (e.g. adrenal growth) is still going on.

The transcriptional variability and post-translational maturation of neuropeptides lead to a diversity of peptides in the nervous system which provides the wonderful flexibility that is essential to biological adaptation and survival of the living organism. In particular, we would like to focus attention on a key element in this mechanism: the prohormone-processing convertases recently isolated and characterized in our laboratory (14).

PRO-OPIOMELANOCORTIN

In 1977, Mains and coworkers, using sodium dodecyl sulfate gel fractionation, isolated a glycoprotein of MW 31,000 containing both ACTH and β-LPH immunoreactive peptides (15). In 1979 Nakanishi cloned and sequenced the cDNA of the bovine intermediate lobe POMC (16). Subsequent studies have determined the structure of human (17), mouse (18), rat (19), and porcine (20) precursors.

Studies on POMC genetic structure in the lower species have suggested that the overall structure and organization of the POMC gene and its products are conserved throughout evolution. The common features of POMC are a 26 amino acid signal peptide followed by an N-terminal peptide (pro-γ-MSH) preceding the ACTH sequence followed by the endorphin-containing sequence, β-LPH. The prohormone molecule also

contains four cysteine residues at the N-terminus, most probably for maintaining correct conformation via disulfide bridges (Figure 2).

The POMC model of neurohormone maturation was also the very first example of a common precursor giving rise to biologically active end-products with entirely different functions. ACTH increases the secretion of adrenocortical steroids, MSH causes dispersal of melanin pigment in melanocytes, β-LPH has some limited lipotropic activity; β-END exerts strong endogenous opioid activity via binding to opiate receptors in the central nervous system. One question remains: are other POMC fragments biologically active?

THE ENIGMA OF ADRENAL GROWTH: a field for possible functional diversity of POMC peptides

Relatively little is known about the exact mechanism of adrenal growth. Even in the era of sophisticated laboratory techniques (RIA) and of discovery of new important elements in ACTH regulation (CRF), the establishment of a precise physiopathological mechanism for Cushing's syndrome remains a major challenge.

Long before the ACTH molecule was isolated in pure form and chemically characterized, its adrenocorticotropic activity was extensively studied. Much slower to be revealed was its possible trophic growth-promoting activities on the adrenal gland. During the course of its isolation, partially purified pituitary extracts, though weak in corticotrophic activity as

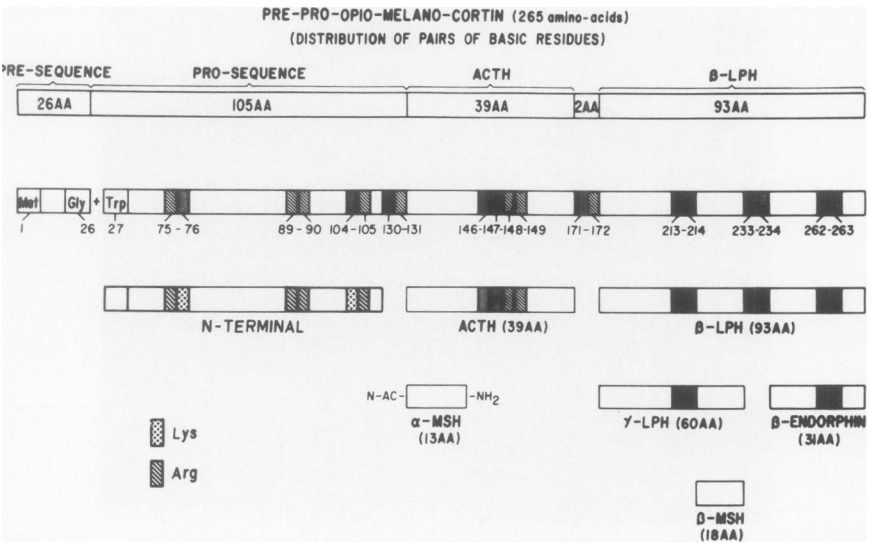

FIG. 2. The structure of pre-pro-opiomelanocortin.

measured by adrenal ascorbic acid depletion, were found to be able to increase the weight and size of the adrenal cortex (21). Further purification allowed the adrenal ascorbic acid depleting activity to be distinguished from an adrenal weight increasing activity (22) and adrenal weight maintenance activity. This latter was first detected by Christy's group (23) in the plasma of patients with Cushing's syndrome due to bilateral adrenal hyperplasia.

In fetal life, the human adrenal glands grow rapidly (from 100 mg to 2 g) 10–20 weeks after gestation (24) by an increase in size of the fetal and definitive zones (Figure 3). After 20 weeks the definitive zone grows much more extensively than the fetal zone. Several factors are responsible for the striking growth and development of the fetal adrenal gland. Fibroblast growth factor (FGF) stimulates mitogenesis at this stage of development while steroidogenesis is maintained (25). It was shown that FGF mRNA is expressed in fetal adrenals and it can be stimulated by adrenocorticotropin (ACTH) (26). In addition, mRNAs for insulin-like growth factors (IGF) I and II were found in fetal adrenals (27). Furthermore, epidermal growth factor (EGF) also can stimulate adrenal growth and EGF receptors also were demonstrated in the fetal gland (28). Numerous mitogens probably play a key role in fetal adrenal growth, but the seminal experiments of Jost (29) showing that anencephalic fetuses have hypoplastic adrenals suggests that the hypophysis also plays a significant role.

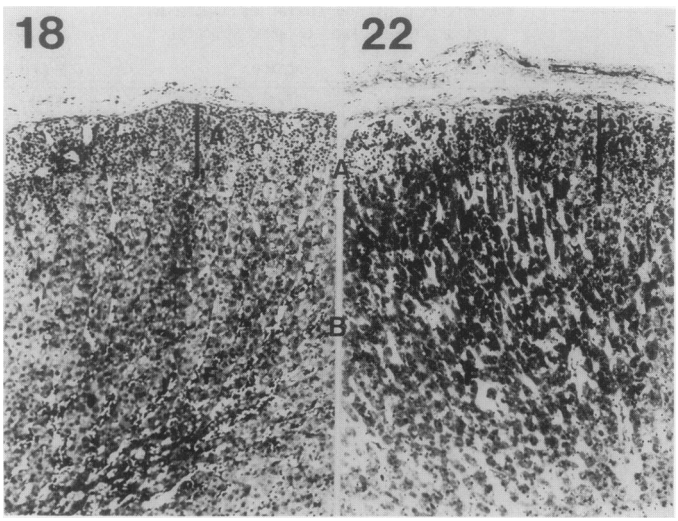

FIG. 3. Histological structure of the fetal adrenal (18 and 22 weeks of gestation). Fetal zone: A, definitive zone: B.

As more work evolved, it became clear that, although ACTH induces the secretion of steroids from a responsive adrenal, it is rather ineffective in stimulating mitotic activity in the adrenals of hypophysectomized rats (30, 31). On the other hand, pituitary growth hormone preparations induced a marked stimulation of cell division with relatively little effect on adrenal weight. But when ACTH and growth hormone are administered together there is a synergistic increase in adrenal weight (31).

In the 1950's histological studies indicated that ACTH causes an increase in adrenal weight by cell hypertrophy rather than hyperplasia. Subsequent biochemical investigations of the 1960's (32–35) showed a role for ACTH in the proliferation of the adrenocortical cells as well. In view of this controversy, further complicated by contradictory results from tumor tissue materials (36), more recent work was carried out on normal adrenal cells in culture. In monolayer culture ACTH does not stimulate DNA synthesis in normal adrenal cells; on the contrary, ACTH often appeared to inhibit DNA synthesis (37, 38). Subsequent *in vivo* studies, in which ACTH antiserum was applied in order to deplete plasma ACTH (39), reaffirmed the fact that ACTH has a trophic action solely directed to the induction and maintenance of the steroidogenic capacity of these cells while adrenocortical cell proliferation must be induced by factors other than ACTH.

Over the past decade, extensive research has been carried out to investigate the non-ACTH components of adrenal growth promoting activity. Although a number of factors of pituitary origin (e.g. vasopressin, growth hormone, etc.) have been suggested as specific adrenocortical mitogens (40, 41), none of them could prevent the adrenal atrophy caused by chronic glucocorticoid administration which affects selectively the hypothalamo-pituitary-adrenal axis. Furthermore, experimental and clinical pituitary-dependent adrenal hyperplasia is extensively linked to the increased corticotropic activity (42, 43). These data have focused the attention of many groups on a putative adrenal mitogen(s), most likely derived from pro-opiomelanocortin (POMC).

Studies of Vinson and coworkers (44) have proved that rat adrenal zona glomerulosa cells show specific sensitivity to stimulation by α-MSH. Other ACTH-derived peptides have also been investigated for their specificity of actions on rat adrenocortical cells. These data suggest that the ACTH 18-24 region contains the signal for the stimulation of the zona fasciculata and zona reticularis while the ACTH 1-13 region shows specificity for the zona glomerulosa response (45). The effect of the long term administration of α-MSH in experimental animals suggests that this treatment can induce a remarkable hypertrophy of the zona glomerulosa cells, mainly by the increase in the volume of mitochondrial compartment and the proliferation of the smooth endoplasmic reticulum

along with an increased secretion of aldosterone. These results suggest that α-MSH enhances not only aldosterone output, but also the growth and steroidogenic capacity of the zona glomerulosa cells (46).

ACTH, β-LPH, β-END, α- and β-MSH derive from a common precursor in the anterior and intermediate lobe of the pituitary (5, 7). Advances in recombinant DNA technology and protein purification have facilitated studies of genes encoding for polypeptide hormone precursors. The mRNA sequence of bovine pituitary pre-POMC was determined in 1979 by Nakanishi et al. (16), and this was followed by reports on DNA sequences of genes for human and rat POMC (17, 19). From these results, it became apparent that a sequence showing extensive homology with α- and β-MSH exists in the N-terminal part of POMC, this was named γ-MSH. At exactly the same time, we isolated the secretory product (47) which is a truncated fragment of the N-terminal sequence of POMC (N-POMC), leaving a short segment (human joining peptide, HJP) which links it to ACTH. Structural analysis revealed that N-POMC is a glycopeptide of MW 12,000 (12 KD) with two sites of glycosylation at Thr-45 and Asn-65 and two disulfide bridges (Figure 4). This peptide was shown to stimulate the release of aldosterone from human adrenal tumor cells (47). Using a sensitive and specific radioimmunoassay (RIA), we demonstrated that N-POMC, ACTH, and β-LPH are secreted in equimolar quantities from human anterior pituitaries *in vitro* (48). Subsequent studies using RIA for both N-POMC and γ-3-MSH (N-POMC 50-76) on the molecular forms of secreted and circulating N-POMC showed that there is only one predominant form of N-POMC (authentic, 12 KD), in normal subjects, patients with Nelson's syndrome and with ectopic ACTH secreting tumors (49, 50) providing no evidence that the

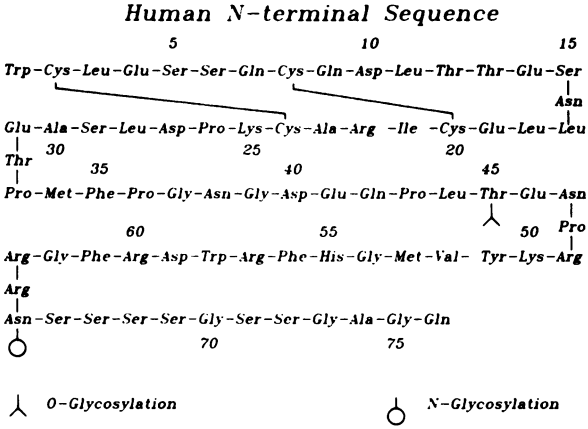

FIG. 4. Amino acid sequence of the N-terminus of human POMC.

N-terminus of POMC undergoes further processing before secretion. Animal experiments showed, however, that as well as removing the joining peptide, the neurointermediate lobe of the pituitary is capable of cleaving most of the paired basic residues of POMC to generate smaller peptides including N-POMC 1-49/50 and Lys-γ-3-MSH (51).

The major source of POMC-derived peptides is the pituitary, but immunoreactive peptides related to POMC can also be detected in other than the pituitary tissues such as gastric mucosa, brain, lung, placenta, and ovaries (52–56). By using radioimmunological detection, we have demonstrated that the human adrenal medulla contains a considerable amount of N-POMC and that the molecule is probably further processed in this tissue (57).

The biological role for N-POMC has been studied extensively. Investigations by different groups showed that γ-MSH probably has no steroidogenic effect *in vivo* (58, 59). However, early studies suggested that intact N-POMC can potentiate the effect of ACTH on isolated superfused rat and human adrenocortical cells to secrete corticosterone/cortisol and aldosterone, so that N-POMC also could modulate ACTH-induced steroidogenesis (60). Intact N-POMC exerts its effect by increasing RNA synthesis in the adrenal cortex (61). However, N-POMC 1-76 is not able to stimulate DNA synthesis *in vitro* and to increase mitogenesis *in vivo,* while N-POMC 1-28 and N-POMC 2-59 can cause significant increase in these activities (62). The exact mechanism for cleavage of N-POMC to express this mitogenic activity is not yet known. The elegant studies of Lowry and coworkers (61) using specific antisera against N-POMC 1-28 and γ-3-MSH showed that N-POMC 1-49/50 stimulates adrenal mitogenesis while γ-3-MSH acts as an adrenal hypertrophic hormone. Data obtained from animal experiments using unilateral adrenalectomy suggest that neural mechanisms are involved in initiating post-translational cleavage of N-POMC in order to bring about contralateral compensatory adrenal hyperplasia (63). More recent studies of Estivariz et al. (64) indicate that mitogenically active N-POMC fragments are most probably generated in the anterior lobe and not from the intermediate lobe of the pituitary supporting certain changes in POMC processing to induce adrenal regeneration. Paracrine and/or neural involvement of the adrenal medulla in these mechanisms is quite likely considering its high N-POMC content, the presence there of smaller N-POMC fragments (57), and the close proximity of the target tissue.

In this complex system, the N-terminal region of POMC (including ACTH) contains a variety of peptides capable of affecting the adrenal activity in many ways. N-POMC 1-49/50 and ACTH together stimulate DNA synthesis, while the intact N-POMC 1-76 and γ-3-MSH potentiate

the action of ACTH by causing RNA synthesis. More work has to be done to elucidate the exact interacting mechanisms and the physiological and pathophysiological role of these processes.

It was generally believed that ACTH is the only important factor influencing adrenocortical functions. However, there is a growing body of evidence that adrenal nerves play an important role in modulating the hypothalamo- pituitary-adrenal axis. Animal experiments investigating "compensatory" growth of the remaining (contralateral) gland after unilateral adrenalectomy showed that unilateral growth could be stimulated by sham operation (65). Adrenal growth could be prevented by ipsilateral hypothalamic or contralateral thoracic spinal cord lesions (66). It also was demonstrated that neonatal chemical sympathectomy can inhibit the mitogenic element of the response to unilateral adrenalectomy (67). Splanchnic nerve activity can stimulate adrenomedullary function and also enhance the sensitivity of the cortex to ACTH stimulation (66). Substances produced by the medulla may be carried by a countercurrent blood flow system within the gland (68), but there is little anatomical evidence to suggest that such a system, with specific vessels, exists. The functional connection of the medulla to the adrenal cortex is more likely to be neural.

Although early studies showed that nerve fibres pass directly through the cortex without forming any branching or synapses (69), more recent ones have indicated the presence of nerve terminals within the cortex (70, 71). According to our current knowledge, the adrenal cortex is supplied by two distinct types of nerves. The first set of neurons, with cell bodies outside the gland, reaches the cortical tissue around the blood vessels, and is independent of the splanchnic neural system. The cell bodies of the other set of neurons are situated in the adrenal medulla, supplying both the medulla and the cortex; this second set of neurons is regulated by the splanchnic nervous system (67). These medullo-cortical connections make feasible our hypothesis as to the role of the adrenal medullary N-POMC related peptides (N-POMC 49/50, γ-3-MSH) in generating adrenocortical hyperplasia.

Studies of Phillips and coworkers (72) demonstrated that the extent of compensatory growth of adrenals after unilateral adrenalectomy is a function of the size of the contralateral gland. They also have found that aldosterone (like other mineralocorticoids) has no effect on compensatory adrenal growth. On the other hand, glucocorticoids can prevent this process. Could it be that dexamethasone also inhibits POMC expression in the adrenal medulla? Also, it would be consistent with the intrinsic medullocortical paracrine regulatory model. Dexamethasone was shown to suppress pituitary production of POMC (73) and decrease secretion of

β-END in a manner paralleling the dose-dependent decrease in ACTH secretion (42). It appears reasonable that secretion of other POMC-derived peptides is likewise reduced. Indeed, we have found that dexamethasone suppresses the plasma level of immunoreactive N-POMC (74). In addition, hypophysectomy cannot prevent compensatory adrenal growth, suggesting important roles for mitogens of extrapituitary (e.g. adrenomedullary) sources. Therefore, it is possible that, in hypophysectomized animals, dexamethasone acts at the adrenal POMC intrinsic system to prevent the local production of N-POMC derived mitogens necessary for adrenal growth. Although more work has to be done to prove this hypothesis, we conclude that POMC contains, besides fragments known to have ACTH/MSH/opioid activities, another fragment (N-POMC) which could well have the long suspected adrenal growth promoting activity, or at least be a cofactor for this important process. Thus, studies searching for biological functions of the so-called inactive fragments of proneuropeptides are worth pursuing.

FUNCTIONAL DIVERSITY OF NEUROPEPTIDES IN THE BRAIN

The CNS is a cell system of great complexity and a great deal of new information about it is being gathered by the application of the current repertoire of molecular biology techniques. We would like to illustrate how nerve cells are able to display various forms of functional diversity by focusing on the molecular level.

1. Differential splicing

This phenomenon was first seen with the calcitonin (CT) gene (75). After cloning CT-related mRNAs, the gene was shown to give rise by translation to peptides whose sequences were both identical and at variance with those found previously: a new peptide (calcitonin gene related peptide, CGRP) was discovered. In parafollicular cells of the thyroid, the mRNAs are transcribed by splicing DNA genomic elements to give a specific mRNA whose composition depends on the cellular milieu (Figure 5). The CT gene has two exons coding for a sequence common to both calcitonin and CGRP precursors. In the thyroid the CT-specific exon is spliced onto the common region exons to generate proCT, while in the hypothalamus CGRP-specific exons are spliced onto the same common region exons to generate proCGRP. Recently a similar mechanism was described for the substance P gene family: the primary structure of two brain substance P precursors revealed the existence of two related products called substance P and substance K, derived by

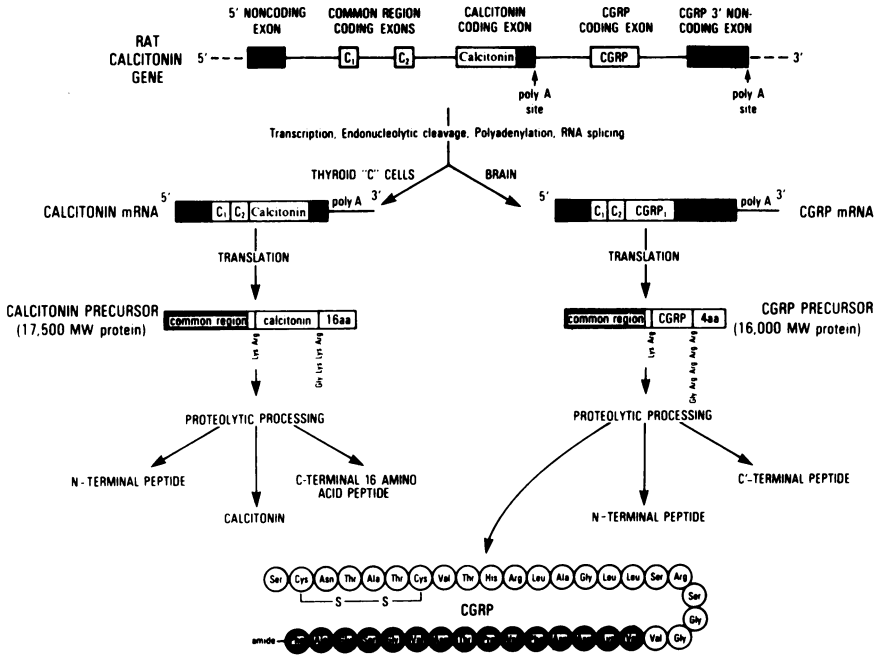

FIG. 5. Differential splicing of the calcitonin gene (Rosenfeld et al, 1983).

alternative splicing from a single gene (76). This is the way by which transcriptional events can regulate the expression of genes so that distinct species of mRNA could be generated from a single gene. Although differential splicing is a great source of gene expression diversity in the immune system, it does not seem to be common to all neuropeptides (unpublished observations).

2. Post-translational processing

Another major route to express molecular diversity is at the post-translational level. By focusing on these steps, we will attempt to illustrate how a cell may greatly enhance its choice of final products by operating through a precursor which, although synthesized as a single polypeptide, contains many different potential active substances joined together.

a) The POMC model

At the time of the discovery of γ-LPH in 1967, we noticed that the sites of cleavage on β-LPH to release both γ-LPH and β-MSH were located at pairs of basic amino acid residues. The biologically inactive

precursors usually undergo a cascade of post-translational events, including proteolytic cleavage and other enzymatic modifications, to yield biologically active end products. However, the presence of two or more basic amino acids often represents a "preferred" site of cleavage. The processing at these sites is not obligatory and there is a tissue-specific pattern of maturation. This specificity might reflect distinct neurotransmitter and endocrine/paracrine biological roles for these substances. It has also been shown that both ACTH and β-MSH can be detected immunohistochemically in one particular cell type (77). The principal end products derived from POMC in the anterior lobe of the pituitary are N-POMC, ACTH and β-LPH, with only limited conversion into smaller molecular forms (β-endorphin). In the intermediate lobe, these products are further processed into α-MSH, CLIP, β-END and γ-LPH. Both α-MSH and β-END are extensively acetylated in the intermediate lobe (Figure 6). There is a significant amount of POMC in the hypothalamus (arcuate nucleus) which is processed as in the intermediate lobe except that the majority of the smaller fragments are not acetylated (78).

b) Other pro-neuropeptides

This converting pattern can be observed for other neuropeptide precursors. The cDNA structure of pro-enkephalin suggests that cleavage

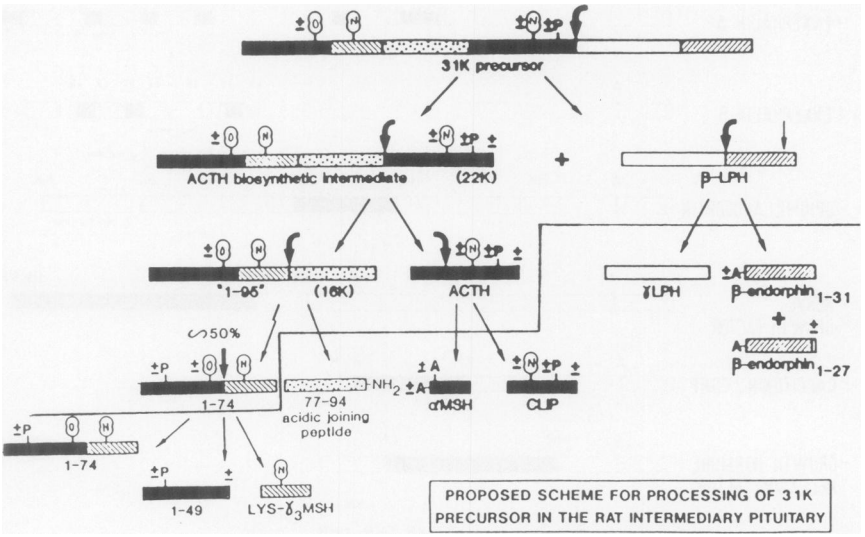

FIG. 6. Processing of pro-opiomelanocortin: further processing of N-POMC, ACTH, and beta-LPH in the intermediate lobe of the pituitary. (O = O-glycosylation, N = N-glycosylation, P = phosphorylation).

at pairs of basic amino acids might give rise to four molecules of Met-enkephalin, one of Leu-enkephalin, a Met-enkephalin heptapeptide and a Met-enkephalin octapeptide, and several other peptides (Figure 7). Extensive work has shown a picture even more complicated than predicted. The conversion of pro-enkephalin proceeds very differently in the adrenal and in the brain. Prodynorphin (also known as prepro-enkeph-

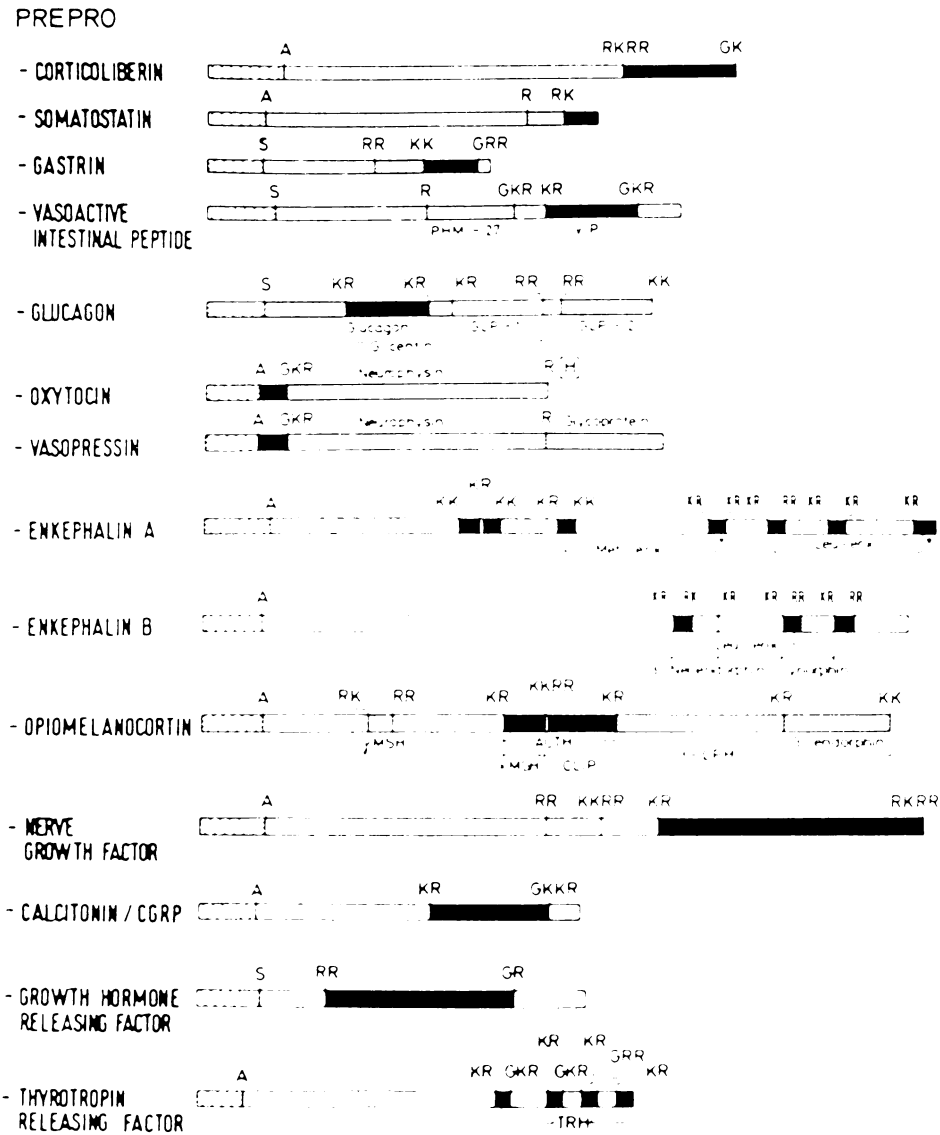

FIG. 7. Structures of precursors for the known neuropeptides.

alin B) gives rise to the products dynorphin A, dynorphin B, α- and β-neoendorphins (79). Pro-GRF consists of 44 amino acids (43 in rat) and fragments of 1-40 and 1-37 have been found in pancreatic tumors and hypothalamic tissue extracts (80); possible further processing is still an open question. Recent studies have provided information about the TRH precursor (81). The cDNA sequence predicts a protein sequence which contains five copies of the Gln-His-Pro-Gly sequence flanked by pairs of basic amino acids (Figure 8). Other potential cleavage sites elsewhere in the pro-TRH will produce other non-TRH peptides, which will be secreted and might have biological activities. Within the GnRH prohormone, the decapeptide is flanked by a signal sequence and a Gly-Lys-Arg site at its C-terminal. The precursor also contains a 56-peptide demarcated by pairs of basic amino acids.

The processing very often includes transformations until the final mature and active end product is produced. The various modifications take place in different cell locations or organelles. However, the key modification event involved in the conversion of a prohormone to its active components is cleavage of the peptide chain at the appropriate site(s). In fact, it is the *sine qua non* chemical event of the whole process. Thus the cleavage enzyme(s), also called convertases, become the key

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    AGCACAGAGCAGCACAAGGACACACTCTGCATATTGTGCTGCCGGACAAGGAGGTGACAGCCAGTCAGGCTGAGACAAAGGA
                          109
ACTTCCAGACCTCTGACAGCAGGAAAG ATG GTG TCT GTC TGG TGG TTG CTG CTT CTC GGT ACA ACC GTA TCT
                            Met-Val-Ser-Val-Trp-Trp-Leu-Leu-Leu-Leu-Gly-Thr-Thr-Val-Ser-
                             1                                                        15
CAC ATG GTG CAC ACA CAA GAG CAG CCT TTA CTG GAG GAG GAC ACA GCA CCA TTA GAT GAC TCG GAT
-His-Met-Val-His-Thr-Gln-Glu-Gln-Pro-Leu-Leu-Glu-Glu-Asp-Thr-Ala-Pro-Leu-Asp-Asp-Ser-Asp-

GTT CTT GAG AAA GCC AAA GGT ATC CTG ATC CGC AGT ATC CTG GAG GGA TTT CAA GAA GGG CAA CAA
-Val-Leu-Glu-Lys-Ala-Lys-Gly-Ile-Leu-Ile-Arg-Ser-Ile-Leu-Glu-Gly-Phe-Gln-Glu-Gly-Gln-Gln-

AAC AAT AGA GAT CTA CCA GAT GCA ATG GAA ATT ATA TCT AAG CGC CAG CAC CCA GGG AAA CGA TTC
-Asn-Asn-Arg-Asp-Leu-Pro-Asp-Ala-Met-Glu-Ile-Ile-Ser-Lys-Arg-Gln-His-Pro-Gly-Lys-Arg-Phe-

CAG GAG GAG ATA GAA AAG AGA CAA CAC CCT GGA AAG AGG GAT CTG GAA GAT CTG AAT CTA GAG CTT
-Gln-Glu-Glu-Ile-Glu-Lys-Arg-Gln-His-Pro-Gly-Lys-Arg-Asp-Leu-Glu-Asp-Leu-Asn-Leu-Glu-Leu-
                                                                                  478
TCC AAA AGG CAA CAC CCC GGA AGA AGA TTT GTG GAT GAT GTA GAG AAG AGG CAA CAT CCA CCCCC...
-Ser-Lys-Arg-Gln-His-Pro-Gly-Arg-Arg-Phe-Val-Asp-Asp-Val-Glu-Lys-Arg-Gln-His-Pro .....
                                                                                 123
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FIG. 8. The coding region of pre-pro-TRH cDNA (Richter and Kreil, 1984).

element in the capacity of the brain and the nervous system to produce thousands of different molecules.

THE CONVERTASES: the elusive enzymes come of age?

Although the precursor theory was proposed in 1967 by Steiner for insulin (82) and by us for the β-LPH model (2), the whole story of convertases has not yet been completely unraveled.

The classical and predominant sites of precursor cleavage are at positions containing two or more consecutive basic amino acids. The configuration most often observed is a single pair of basic amino acids. The most frequently seen combination is Lys-Arg, which nearly always leads to cleavage. However, processing may also occur at the Arg-Arg, Lys-Lys or Arg-Lys sites. Theoretical analysis studies suggest that the processing sequences cleaved *in vivo* are located inside regions with a high β-turn potential, or else immediately adjacent to these structures (83). Experimental replacement of the Arg or Lys residues in prohormones during biosynthesis can prevent cleavage of these substances (84). However, in many cases cleavage of precursor C-terminals to single Arg or Lys residues can be found (chicken proalbumin, propressophysin, proCCK, chromogranin A, proANF, etc.) (Figure 9). Although there is no distinct consensus element which characterizes monobasic cleavage sites, a proline residue often is found either just before or just after the basic residue designating the processing cleavage site. It has been suggested also that the conformation of the polypeptide backbone of the precursor polypeptide is especially important in monobasic processing. Less frequently found sites of processing are Leu-Ala, Leu-Ser, Leu-Leu,

PREDOMINANT SITES OF PRECURSOR CLEAVAGES

PAIRS OF BASIC AMINO ACIDS:	**LYS-ARG**
	ARG-ARG
	LYS-LYS
	ARG-LYS
SINGLE BASIC AMINO ACID:	**ARG, LYS**
OTHERS:	**LEU-ALA**
	LEU-SER
	LEU-LEU
	VAL-LEU
	ALA-GLY

FIG. 9. Predominant cleavage sites during neuropeptide precursor conversion.

Val-Leu, and Ala-Gly. These kinds of cleavages have been found in relaxin, propressophysin glycopeptide and mellitin.

The fact that neuropeptides are derived from large molecular weight precursors implies the existence of an enzyme or enzyme complex capable of cleaving preferentially at recognized pairs of basic amino acids. Although an extensive search for the appropriate proteolytic specificity has been going on for two decades, no authentic enzyme has yet been purified and characterized. The major difficulty is the lack of an appropriate assay system and the limited quantity present in the neural and endocrine tissues. Potential convertases have been reported from eukaryotes and the candidates proposed encompassed several classes of enzyme activity.

Findings from experiments with the endocrine pancreas granule system indicate that thiol proteases having properties similar to cathepsin B may represent an important class of processing enzymes (85). These putative enzymes might be involved in the maturation of prosomatostatin, proglucagon, and proinsulin in secretory granules. Cathepsin B and its larger molecular weight form have been suggested to play a role in the proinsulin processing that takes place in mature secretory granules. Work from the laboratory of Loh (86) demonstrated that an aspartyl protease with a pH optimum of 4–5 could be isolated from pituitary secretory granules and could be responsible for the *in vivo* processing of POMC, propressophysin and pro-oxyphysin at pairs of basic residues. Others (87) suggested the presence of a metalloprotease with a neutral pH optimum in secretory granule pools of the hypothalamus, which cleaved pro-oxytocin and pro-somatostatin at the pairs of basic residues Lys-Arg and Arg-Lys. Moreover, serine protease converting activities with a basic pH optimum have been identified in secretory granules of the parathyroid, pituitary, and adrenal glands. One of the activities reported by Powers (88) seems to be glandular kallikrein, whereas the other, called kallikrein A, resembles in many respects the serine protease recently isolated in our laboratory (89). We have shown that the latter enzyme isolated from porcine and human pituitary is structurally identical to plasma prekallikrein. The enzyme cleaves POMC, proinsulin, proenkephalin and proANF. To date, many other potential maturation enzymes have been proposed, although a fully purified and characterized candidate has yet to be reported.

The major limitation of this approach has been the relatively low abundance of the physiologically relevant processing enzymes and the observation that a number of potential convertases can perform specific cleavages at pairs of basic residues *in vitro*. However, in the absence of purified and molecularly identified processing enzymes that can be kinetically studied *in vitro*, gene transfer technology accompanied by site-directed mutagenesis has allowed the characterization of the substrate

specificity of the prohormone processing machinery *in vivo* in numerous cell lines. This new approach has permitted the identification of enzyme-coding genetic structures present within some of the cell lines.

Until lately, the only prohormone convertase sufficiently characterized was the yeast Kex2 gene product which has been characterized at the molecular level (90). Kex2 is a Ca2+ dependent serine proteinase sharing remarkable similarities with bacterial subtilisins. By using gene transfer techniques, this enzyme was shown also to confer converting capability on cell lines which normally do not mature prohormones at pairs of basic residues (91). More recently, it was proposed also that a human furin gene product coding a hepatic 4.3 kb mRNA could represent the mammalian counterpart of Kex2 (92). Using polymerase chain reaction (PCR) technology, we were able to isolate and clone two highly homologous but distinct cDNA structures (called mouse pituitary convertase, MPC 1 and 2) from a mouse pituitary cDNA library (14). These products were shown to be similar to Kex2 and human furin. Independently from us and at the same time (as for the prohormone theory 23 years ago), Steiner and associates (93) isolated the human equivalent of our MPC2 from insulinoma. Figure 10 compares the amino acid sequences of MPC1, MPC2, Kex2 and furin. Both MPC1 and MPC2 show remarkable similarity to Kex2 and furin, especially around the catalytic triad of Asp-His-Ser. Furthermore, if one compares these active site sequences with the subtilisin family of proteases, it is highly probable that MPC1 and MPC2, like Kex2 and furin, are also serine proteases belonging to this important enzyme family (Figure 11). Preliminary results of transfection studies in our group indicate characteristic *in vivo* enzymatic activity of MPC1 and MPC2 translational products.

In Figure 12 a Northern blot comparison of the murine tissue and cellular expression of MPC1 and MPC2 is shown. The mRNA transcripts are predominantly about 3 kbp in size, while in brain an approximately 4.8 kbp transcript is expressed in equal quantity. MPC1 is most abundant in the pituitary, followed by the adrenals and hypothalamus. A pituitary tumor cell line (AtT20) seems to contain abundant MPC1 mRNA while being less abundant in mouse insulinoma cells. No transcripts were detected in tissues and cells of non-endocrine origin. In contrast to MPC1, MPC2 mRNAs can barely be detected in adrenals and the pituitary, the richest sources were the hypothalamus and the brain. The smaller form of MPC2 is most abundant in the β-cells of the pancreas. The complete nucleotide sequence of MPC1 and MPC2 predicts 753- and 637-residue proteins, respectively, with three potential N-glycosylation sites. No indication of the existence of a transmembrane domain has been found in these two products. The soluble carboxypeptidase E (CPE)

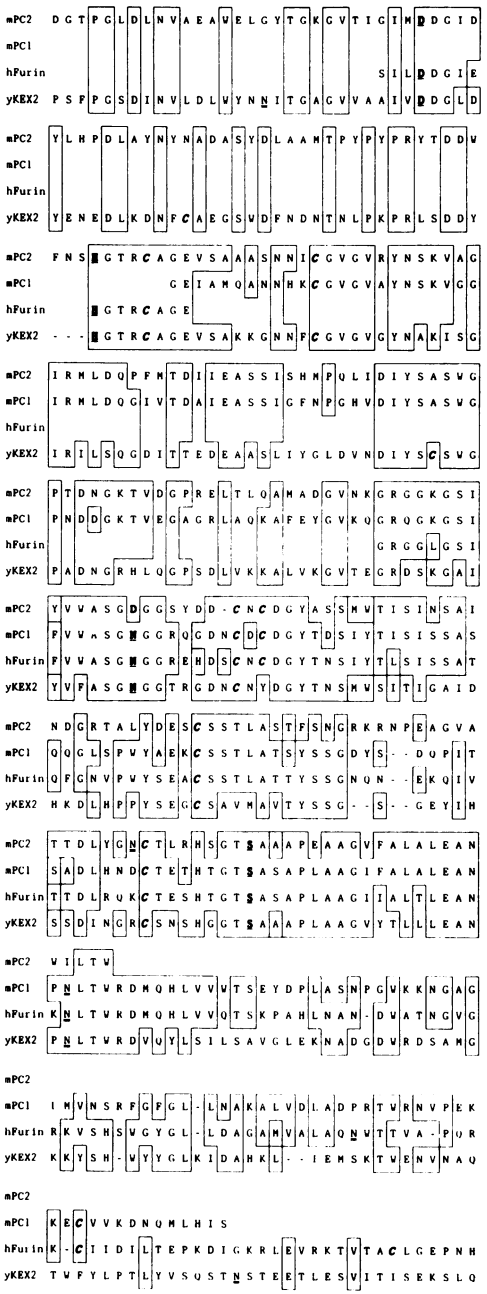

FIG. 10. Comparison of the amino acid sequences of MPC1, MPC2, KEX2, and human furin (active site residues are marked).

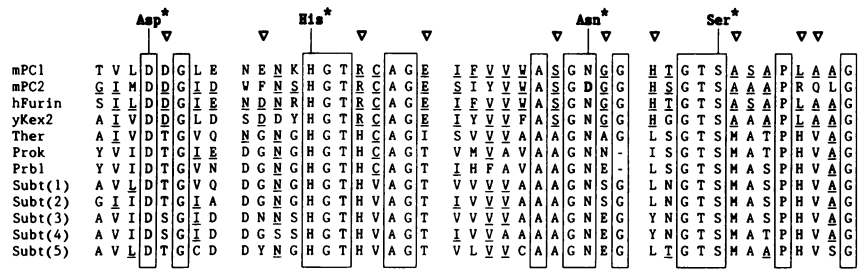

FIG. 11. Conservation of active sites among members of the subtilisin family (inverted triangles identify the amino acids found only in eukaryotes).

participating in peptide processing was shown recently to contain an amphipathic α-helical sequence within its C-terminal segment; this structure is probably responsible for its observed pH-dependent association with membranes. A similar analysis applied to MPC1 and MPC2 showed that they also contain C-terminal amphipathic domains in which hydrophobic and hydrophilic residues align on opposite sides of the helix (Figure 13).

In situ hybridization (Figure 14) with MPC1 showed labeling in 20% of the cells of the anterior pituitary and in almost all the cells of the intermediate lobe. In the hypothalamus, a particular enrichment in the supraoptic nucleus was seen. One can notice also that the supportive cells (endothelium, mast cells, blood cells, pituicytes and glial network) are not stained. We have found labeled cells in the adrenal medulla, but not in the cortex. A substantially different distribution pattern of MPC2 mRNA was observed. Within the pituitary, high intensity specific labeling was generated in all melanotropes of the intermediate lobe. No detectable MPC2 was found in either the anterior lobe of the pituitary or in the adrenal medulla. In the brain, moderate to high density labeling was seen to be widely distributed in all six layers of the cortex, hippocampus, and in different thalamic areas. Less dense labeling was shown in the hypothalamus such as in the nucleus supraopticus and nucleus paraventricularis. A few labeled neurons were observed in the posterior hypothalamus (ventral border of the nucleus ventromedialis). Positive neural cells were displayed in layers III to IX of the spinal cord. Figure

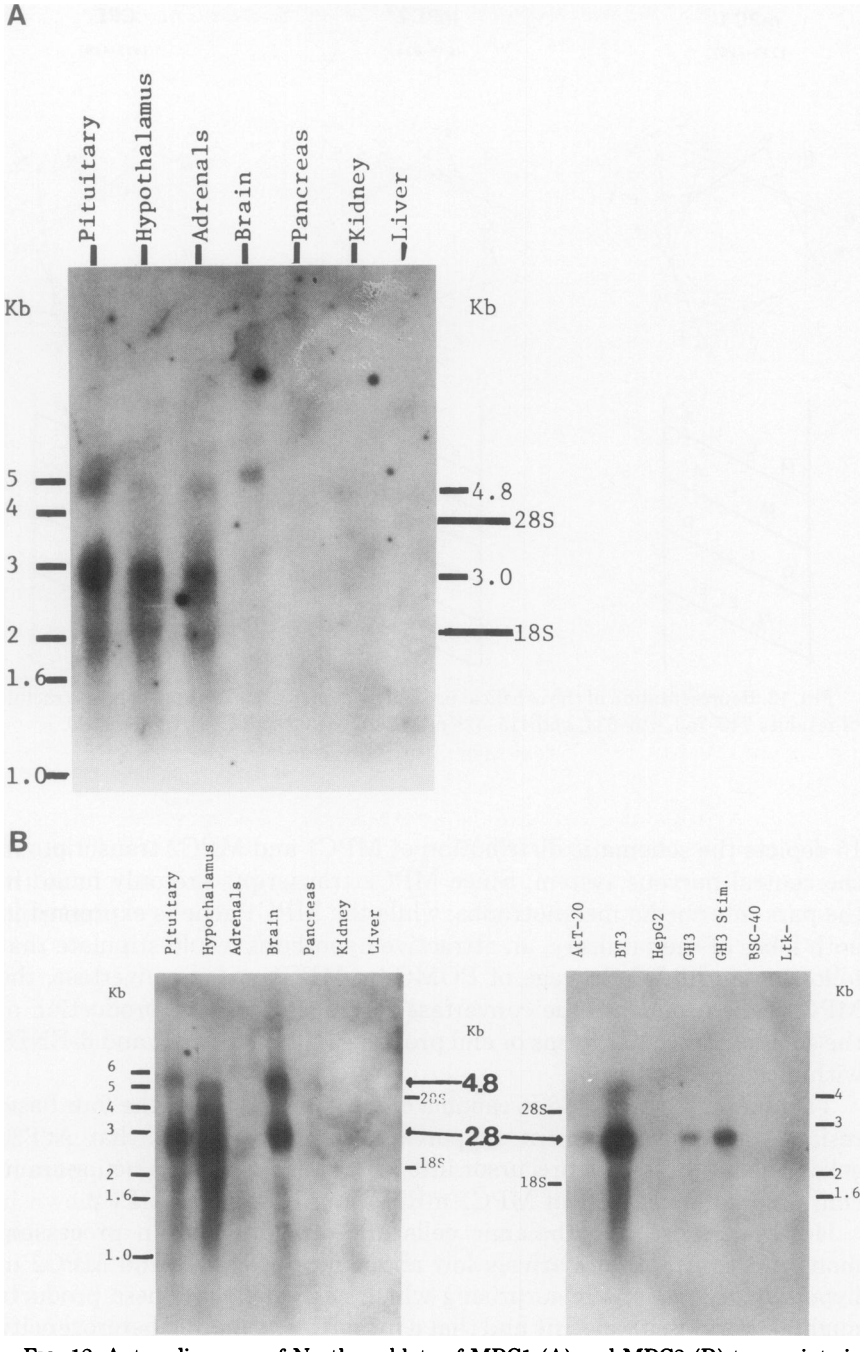

FIG. 12. Autoradiograms of Northern blots of MPC1 (A) and MPC2 (B) transcripts in tissues and cells.

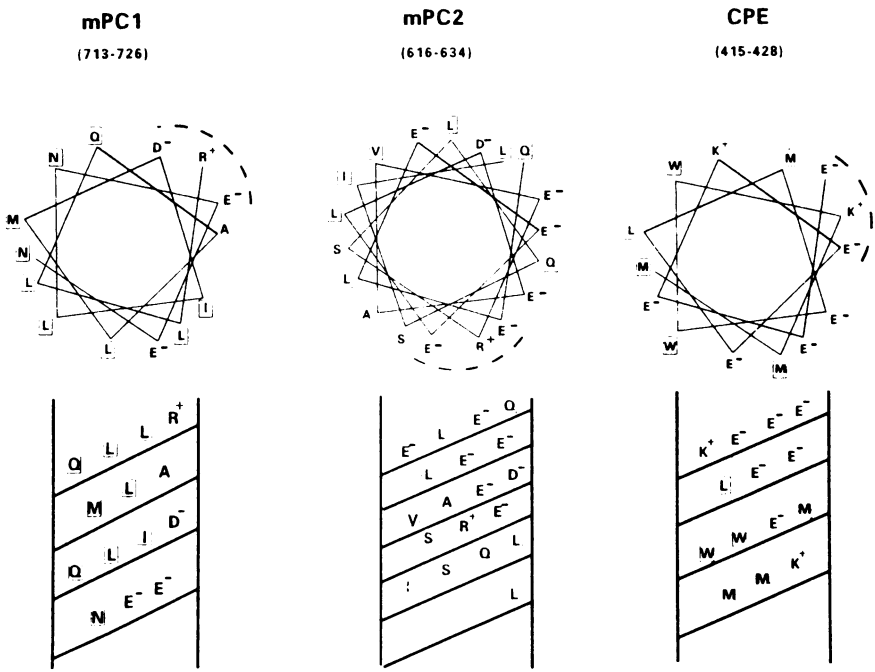

FIG. 13. Representation of the α-helical net and axial projection of the α-helical structure of residues 740–753, 616–634, and 415–428 of MPC1, MPC2 and CPE, respectively.

15 depicts the schematic distribution of MPC1 and MPC2 transcripts in the central nervous system. Since MPC2 transcripts are only found in the pars intermedia melanotrophs, while the MPC1 gene is expressed in both lobes of the pituitary, an attractive hypothesis would stipulate that following an initial cleavage of POMC by MPC1 coded convertase, the MPC2 gene product is the convertase responsible for the production of the smaller molecular forms of end products (CLIP, α-MSH and β-END) within the melanotrophs.

This implies that MPC2 is capable of cleaving ACTH at the four basic residues. Another argument supporting our hypothesis is that AtT20 cells, which process the precursor into ACTH as a final product, seem to contain a large quantity of MPC1 mRNA, but little or no MPC2.

However, since hypothalamic cells are very efficient in processing many precursors, the relatively low abundance of MPC1 and MPC2 in hypothalamic regions is surprising which suggests, that these products might be very tissue specific and that a hitherto unexpected heterogeneity

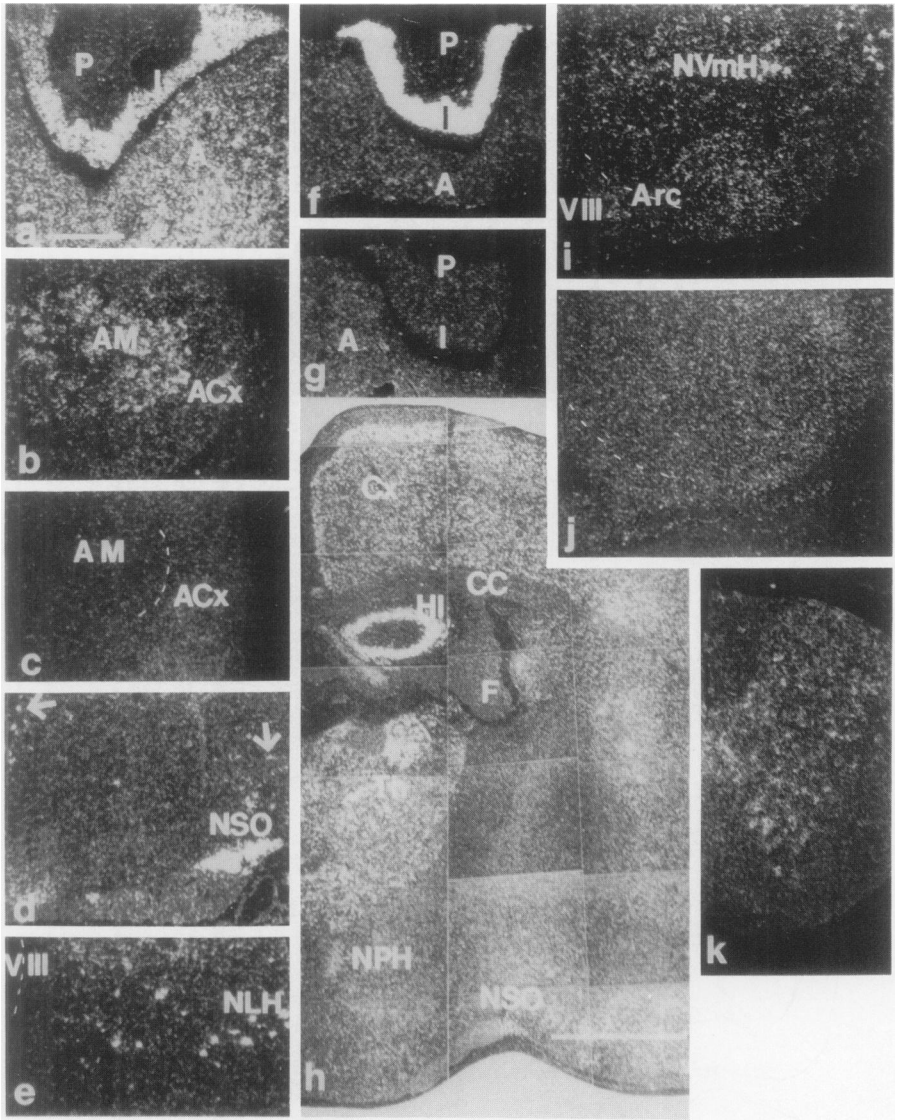

FIG. 14. *In situ* hybridization. MPC1: (a) pituitary gland, intermediate lobe (I), anterior lobe (A), posterior lobe (P); (b) adrenal gland, medulla (AM), cortex (ACx), control hybridization with sense riboprobe (c); (d) hypothalamus, nucleus supraopticus (NSO), labeled neurons (arrows) in the nucleus periventricularis; (e) nucleus lateralis (NLH); MPC2: (f) pituitary gland, intermediate lobe (I), anterior lobe (A), posterior lobe (P); sense riboprobe control (g); (h) brain, moderate to high intensity labeling in all six layers of the cortex (Cx), hippocampus (hi), and in different thalamic areas (Th); less dense labeling in the hypothalamus such as nucleus supraopticus (NSO) and nucleus paraventricularis (NPH); no labeling in corporis callosi (CC) and fimbria (F); (i) rarely labeled neurons in the posterior hypothalamus located in the nucleus ventromedialis (NVmH); no label in the arcuate nucleus (Arc); V III = third ventricle; (j) sense riboprobe control; (k) cross section through the cervical segment of the spinal cord: labeled neurons in layers III to IX with ventral predominance.

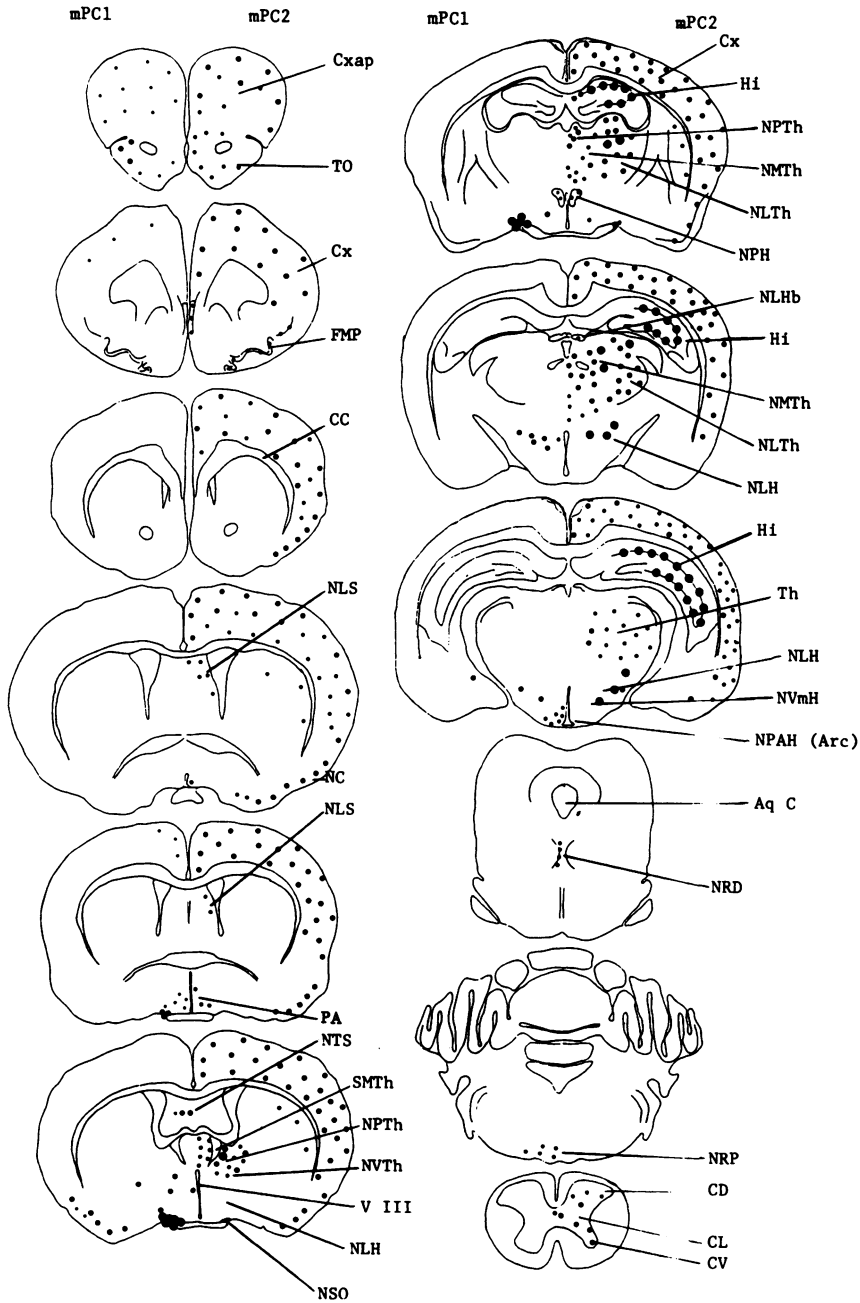

FIG. 15. Brain maps of MPC1 and MPC2. Abbreviations: Aqc aqueductus cerebri, CC corporis callosi, CD cornu dorsalis, CL cornu laterale, CV cornu ventrale, Cx cortex cerebri,

in the nature of processing convertases exist in this region. In contrast to MPC1, MPC2 has been found in the cortex and subcortical areas of the brain suggesting an involvement of MPC2 in the processing of proCCK, proVIP and possibly other active substances. Furthermore, it is also surprising that very little message is found in the arcuate nucleus known to process POMC into smaller fragments. It would be interesting to elucidate whether it is caused by other convertases belonging to this family of subtilisin like enzymes.

Figure 16 shows the chromosomal localization of MPC1 and MPC2: the MPC1 was localized in the C1-C3 region of chromosome 13, whereas MPC2 hybridization is seen in the F3-H2 region of chromosome 2 in mouse. These data suggest that these molecules are not results of a recent gene duplication event.

However, it is known also that structural genes for enzymes catalysing subsequent steps in a given metabolic pathway are usually not syntenic (94). This would fit with our model proposed for MPC1 and MPC2 within the intermediate lobe of the pituitary gland.

Processing of polypeptide hormones is known to start at the trans-Golgi network and continues within the secretory granules (95). This delicate process requires the contribution of various gene products including the convertases. The number and interrelation of these proteases is not yet known. Other biosynthetic and gene transfer experiments indicate that cells vary widely in their processing mechanisms for prohormone maturation. No clear explanation for such tissue- and cell-specific processing has been provided so far. We hope that recent work carried out in our group will help to answer some of these questions.

In conclusion, the functional diversity of the brain is provided by four major sources: first, the variety of transcription of neuropeptide precursor genes in different regions of the central nervous system. It has been suggested that more genes are required for the function of brain than for all the other tissues and organs combined (96). More diverse population of gene products is consistent with the extensive cellular heterogeneity and differentiation of various cell types found in the brain and the

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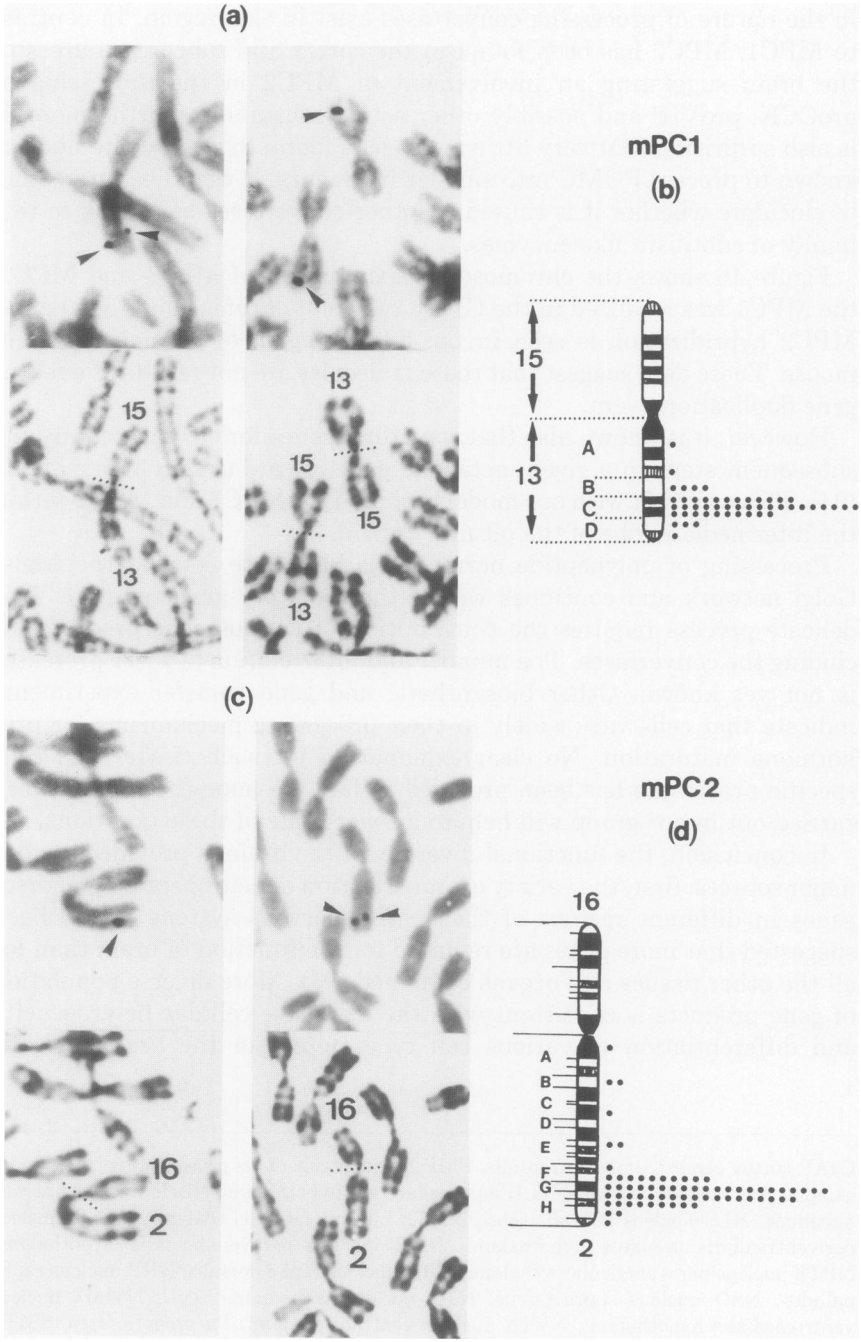
CxAP cortex cerebri, area prefrontalis, FMP fasciculus medialis prosencephali, Hi hippocampus, NC, nucleus caudatus, NLH nucleus lateralis hypothalami, NLHb nucleus lateralis habenuae, NLS nucleus lateralis septi, NMTh nucleus lateralis thalami, NPAH nucleus periventricularis arcuatus hypothalami, NPH nucleus paraventricularis hypothalami, NPTh nucleus paraventricularis thalami, NRD nucleus raphe dorsalis, NRP nucleus raphe pallidus, NSO nucleus supraopticus, NTS nucleus triangularis septi, NVmH nucleus ventromedialis hypothalami, NVTh nucleus ventralis thalami, Pa preoptic area, SMTh stria medullaris thalami, Th thalamus, TO tractus olfactorius, V III ventriculus tertius.


(a)
15
13
13
15
15
13
mPC1
(b)
15
13
A
B
C
D
(c)
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2
mPC2
(d)
16
A
B
C
D
E
F
G
H
2

complexity of brain mRNA sequences is remarkably greater than that of other tissues; hybridization studies on RNA of nuclear and polysomal origin and of polysomes showed about 150,000 distinct sequences (97). Second, the conversion of a precursor to its active fragments. The number of potential peptide products which can be generated from the conversion of a precursor by cleavage at pairs of basic residues at all possible sites can be expressed by the formula of:

$$\sum_{i=1}^{n} (i + 1) = \frac{n(n + 3)}{2}$$

as i varies from 1 to n where n represents the number of possible cleavage sites. For example, the cleavage of POMC can potentially give rise to 54 different peptides. Thus, a single mRNA can magnify its potential diversity 54-fold. Third, as demonstrated by Hökfelt et al. (98, 99) using immunocytochemistry at the electron microscopy level, a single brain cell might contain not only one active neurotransmitter, but a variety of combinations of neurotransmitters and neuropeptides. This co-existence is another way to increase functional diversity and can be formulated as follows: $n!/r!(n - r)!$, or specifically if we chose 2 from 20 neuropeptides, the number of potential combinations $20!/2!18! = 190$. Fourth, the variety of biological functions of the target cells acted on by neuropeptides released from nerve endings. In addition, different receptors via their second messenger systems can also interact on one particular target cell in order to induce delicate changes in function necessary for better adaptation. It is now accepted that receptors (e.g. GABA) also have their own source of diversity (100).

The phenomena we have described, as well as those of which we are not yet aware, all tend towards the amplification of biologically active end products. They also allow us to imagine that brain cells can express a diversity of functions that is beyond anything so far encountered in biology, even exceeding the immense diversity of immunoglobulins.

FIG. 16. Localization of MPC1 and MPC2 genes to mouse chromosomes 13 and 2 respectively, by in situ hybridization. (a,c): pairs of partial WMP mouse metaphases, showing the specific site of hybridization to chromosome 13 (a;MPC1) or chromosome 2 (c;MPC2). Top, arrowheads indicate silver grains on Giemsa-stained chromosomes, after autoradiography. Bottom, chromosomes with silver grains were subsequently identified by R-banding. (b,d): diagram of WMP mouse Rb (13;15, MPC1) or Rb (2,16, MPC2) chromosome, indicating the distribution of labeled sites on either chromosomes 13 (b,MPC1) or on chromosome 2 (d,MPC2).

REFERENCES

1. Chrétien M, Benjannet S, Gossard F, et al. From β-lipotropin to β-endorphin and pro-opiomelanocortin. *Can J Biochem* 1979; 57: 1111.
2. Chrétien M, Li CH. Isolation, purification and characterization of γ-lipotropic hormone from sheep pituitary glands. *Can J Biochem* 1967; 45: 1163.
3. Li CH, Chung D. Isolation and structure of an untriakontapeptide with opiate activity from camel pituitary glands. *Proc Natl Acad Sci USA* 1976; 73: 1145.
4. Orth DN, Nicholson WE, Mitchell WM, et al. ACTH and MSH production by a single cloned mouse pituitary tumor cell line. *Endocrinology* 1973; 92: 385.
5. Lowry PJ, Hope J, Silman RE. The evolution of corticotropin, melanotropin and lipotropin. *Proc of 5th Int Congr Endocr* 1976; 1: 71.
6. Crine P, Gianoulakis C, Seidah NG, et al. Biosynthesis of β-endorphin from β-lipotropin and a larger molecular weight precursor in rat pars intermedia. *Proc Natl Acad Sci USA* 1978; 75: 4719.
7. Roberts JL, Herbert E. Characterization of a common precursor to corticotropin and β-lipotropin: cell free synthesis of the precursor and identification of corticotropin peptides in the molecule. *Proc Natl Acad Sci USA* 1977; 74: 4826.
8. Li CH. β-endorphin: a pituitary peptide with potent morphine-like activity. *Arch Biochem Biophys* 1977; 183: 592.
9. Guillemin R, Ling N, Burqus R, et al. Characterization of endorphins, novel hypothalamic and neurophypophyseal peptides with opiate like activity: evidence that they induce profound behavioral changes. *Psychoneuroendocrinology* 1977; 2: 59.
10. Krieger DT. Pituitary hormones in the brain: what is their function? *Fed Proc* 1980; 39: 2937.
11. Comb M, Seeburg PH, Adelman J, et al. Primary structure of the human Met- and Leu-enkephalin precursor and its mRNA. *Nature* 1982; 295: 663.
12. Kakidani H, Furutani Y, Takahashi H, et al. Cloning and sequence analysis of cDNA for porcine β-neo-endorphin/dynorphin precursor. *Nature* 1982; 298: 245.
13. Chrétien M, Sikstrom R, Lazure C, et al. Expression of the diversity of neural and hormonal peptides via the cleavage of precursor molecules. In: Martinez J, ed. *Peptide hormones as prohormones.* England: Ellis Horwood Ltd; 1989: 1.
14. Seidah NG, Gaspar L, Mion P, et al. cDNA sequence of two distinct pituitary proteins homologous to KEX2 and Furin gene products: tissue specific mRNAs encoding candidates for prohormone processing proteinases. *DNA* 1990; 9: 415.
15. Mains RE, Eipper BA, Ling N. Common precursor to corticotropins and endorphins. *Proc Natl Acad Sci USA* 1977; 74: 3014.
16. Nakanishi S, Inoue A, Kita T, et al. Nucleotide sequence of cloned cDNA for bovine corticotropin β-lipotropin precursor. *Nature* 1979; 278: 423.
17. Chang ACY, Cochet M, Cohen SN. Structural organization of human genomic DNA encoding the proopiomelanocortin peptide. *Proc Natl Acad Sci USA* 1980; 77: 4890.
18. Uhler M, Herbert E. Complete amino acid sequence of mouse proopiomelanocortin derived from the nucleotide sequence of proopiomelanocortin cDNA. *J Biol Chem* 1983; 258: 257.
19. Drouin J, Goodman HM. Most of the coding region of rat ACTH-LPH precursor lacks intervening sequences. *Nature* 1980; 288: 610.
20. Boileau G, Barbeau C, Jeanotte L, et al. Complete structure of the porcine pro-opiomelanocortin mRNA derived from the nucleotide sequence of cloned cDNA. *Nucleic Acids Res* 1983; 11: 8063.
21. Astwood EB. Growth hormone and corticotropin. In: Pincus G, Thimann KV, eds. *The Hormones.* New York: Academic Press; 1955: Vol 3: 235.

22. Dixon HFB, Stack-Dunne MP, Young FG, et al. Chromatography of adrenotropic hormone on ion-exchange columns. *Nature* 1951; 169: 1044.
23. Jailer JW, Longson D, Christy NP. Studies in Cushing's Syndrome. II. Adrenal weight-maintaining activity in the plasma of patients with Cushing's Syndrome. *J Clin Invest* 1957; 36: 1608.
24. Jirasek JE. Development of the genital system and male pseudohermaphroditism Baltimore: Johns Hopkins Press; 1971: 10.
25. Crickard K, Ill CR, Jaffe RB. Control of proliferation of human fetal adrenal cells in vitro. *J Clin Endocrinol Metab* 1981; 53: 790.
26. Jaffe RB, Mulchaney JJ, DiBlasio AM, et al. Peptide regulation of pituitary and target tissue function and growth in the primate fetus. *Recent Prog Horm Res* 1988; 44: 431.
27. Han VKM, D'Ercole AJ, Lund PK. Cellular localization of somatomedin (insulin-like growth factor) messenger RNA in the human fetus. *Science* 1987; 236: 193.
28. Crickard K, Fujii DK, Jaffe RB. Isolation and identification of human fetal adrenal medullary cells in vitro. *J Clin Endocrinol Metab* 1982; 55: 1143.
29. Jost A. Problems of fetal endocrinology: the adrenal glands. *Rec Prog Horm Res* 1966; 22: 541.
30. Cater DB, Stack-Dunne MP. The histological changes in the adrenal of the hypophysectomized rat after treatment with pituitary preparations. *J Pathol Bacteriol* 1955; 66: 119.
31. Cater DB, Stack-Dunne MP. The effects of growth hormone and corticotrophin upon the adrenal weight and adrenocortical mitotic activity in the hypophysectomized rat. *J Endocrinol* 1955; 12: 174.
32. Bransome ED, Reddy WJ. Studies of adrenal nucleic acids: the influence of ACTH, unilateral adrenalectomy and growth hormone upon adrenal RNA and DNA in the dog. *Endocrinology* 1961; 69: 997.
33. Imrie RC, Ramiah TR, Antoni F, et al. The effect of adrenocorticotrophin on the nucleic acid metabolism of the rat adrenal gland. *J Endocrinol* 1965; 32: 302.
34. Masui H, Garren LD. On the mechanism of action of adrenocorticotropic hormone. *J Biol Chem* 1970; 245: 2627.
35. Gill GN. Mechanism of ACTH action. *Metabolism* 1972; 21: 571.
36. Masui H, Garren LD. Inhibition of replication in functional mouse adrenal tumor cells by adrenocorticotropic hormone mediated by adenosine 3′:5′-cyclic monophosphate. *Proc Natl Acad Sci USA* 1971; 68: 3206.
37. Ramachandran J, Lee V. Preparation and properties of the O-nitrophenyl sulphenyl derivative of ACTH: an inhibitor of the lipolytic action of the hormone. *Biochem Biophys Res Commun* 1970; 38: 501.
38. Ramachandran J, Suyama AT. Inhibition of replication of normal adrenocortical cells in culture by adrenocorticotropin. *Proc Natl Acad Sci USA* 1975; 72: 113.
39. Ramachandran J, Kong YC, Liles S. Effects of ACTH and its O-nitrophenyl sulphenyl derivative on adrenocortical function *in vivo*. *Acta Endocrinol* 1976; 82: 587.
40. Payet N, Lehoux JG. A comparative study of the role of vasopressin and ACTH in the regulation of growth and function of rat adrenal glands. *J Steroid Biochem* 1980; 12: 461.
41. Gospodarowicz D, Handley HH. Stimulation of division of Y-1 adrenal cells by a growth factor isolated from bovine pituitary glands. *Endocrinology* 1975; 97: 102.
42. Krieger DT. Physiopathology of Cushing's disease. *Endocrine Rev* 1984; 4: 22.
43. Taki MT, Nickerson PA. Differentiation and proliferation of adrenocortical cells during the early stages of regeneration. *Lab Invest* 1985; 53: 91.
44. Vinson GP, Whitehouse BJ, Thody AJ. α-MSH at physiological concentrations

stimulates "late pathway" steroid products in adrenal zona glomerulosa cells from sodium restricted rats. *Peptides* 1981; 2: 141.

45. Vinson GP, Whitehouse BJ, Bateman A, et al. The actions of N-terminal fragments of corticotrophin on steroidogenesis in dispersed rat adrenal cells in vitro. *J Endocrinol* 1986; 109: 275.
46. Robba C, Rebuffat P, Mazzocchi G, et al. Long-term trophic action of α-melanocyte-stimulating hormone on the zona glomerulosa of the rat adrenal cortex. *Acta Endocrinol* 1986; 112: 404.
47. Seidah NG, Rochemont J, Hamelin J, et al. Primary structure of the major human pituitary pro-opiomelanocortin NH2-terminal glycopeptide. *J Biol Chem* 1981; 256: 7977.
48. Chan JSD, Seidah NG, Chrétien M. Measurement of N-terminal (1-76) of human proopiomelanocortin in human plasma: correlation with adrenocorticotropin. *J Clin Endocrinol Metab* 1983; 56: 791.
49. Gaspar L, Chan JSD, Seidah NG, et al. Urinary molecular forms of human N-terminal of pro-opiomelanocortin: possible deglycosylation and degradation by the kidney. *J Clin Endocrinol Metab* 1984; 59: 614.
50. Gaspar L, Chan JSD, Seidah NG, et al. Characterization of N-terminal fragment of proopiomelanocortin in cerebrospinal fluid. *J Clin Endocrinol Metab* 1987; 65: 198.
51. Estivariz FE, Birch NP, Loh YP. Generation of Lys-γ-3-melanotropin from pro-opiomelanocortin 1-77 by a bovine intermediate lobe secretory vesicle membrane-associated aspartic protease and purified pro-opiomelanocortin converting enzyme. *J Biol Chem* 1989; 264: 17796.
52. Tanaka I, Yoshikatsu N, Kazuwa N, et al. Presence of immunoreactive γ-melanocyte-stimulating hormone, adrenocorticotropin, and β-endorphin in human gastric antral mucosa. *J Clin Endocrinol Metab* 1982; 54: 392.
53. Watson SJ, Akil H, Richard CW, et al. Evidence for two separate opiate peptide neuronal systems and the coexistence of β-lipotropin, β-endorphin and ACTH immunoreactivation in the same hypothalamic neurons. *Nature* 1978; 275: 226.
54. Gewirtz G, Yalow RS. Ectopic ACTH production in carcinoma of the lung. *J Clin Invest* 1974; 53: 1022.
55. Liotta AS, Krieger D. *In vitro* biosynthesis and comparative post translational processing in immunoreactive precursor corticotropin/β-endorphin by human placental and pituitary cells. *Endocrinology* 1980; 106: 1504.
56. Keyzer Y, Lenne F, Massias J-F, et al. Pituitary like proopiomelanocortin transcripts in human Leydig cell tumors. *J Clin Invest* 1990; 86: 871.
57. Gaspar L, Chan JSD, Seidah NG, et al. Peptides related to the N-terminus of pro-opiomelanocortin in the human adrenal medulla. *Clin Invest Med* 1989; 12: 90.
58. Estivariz FE, Iturizza F, McLean C, et al. Stimulation of adrenal mitogenesis by N-terminal proopiocortin peptides. *Nature* 1982; 297: 419.
59. Gullner HG, Nicholson WE, Gill JR, et al. Plasma immunoreactive proopiomelanocortin-derived peptides in patients with primary hyperaldosteronism, idiopathic hyperaldosteronism with bilateral adrenal hyperplasia, and dexamethasone-suppressible hyperaldosteronism. *J Clin Endocrinol Metab* 1983; 56: 853.
60. Al-Dujaili EAS, Hope J, Estivariz FE, et al. Circulating human pituitary pro-γ-melanotropin enhances the adrenal response to ACTH. *Nature* 1981; 291: 156.
61. Lowry PJ, Silas L, McLean C, et al. Pro-γ-melanocyte stimulating hormone cleavage in adrenal gland undergoing compensatory growth. *Nature* 1983; 306: 70.
62. Estivariz FE, Carino M, Lowry PJ, et al. Further evidence that N-terminal proopiomelanocortin peptides are involved in adrenal mitogenesis. *J Endocrinol* 1988; 116: 201.

63. Charlton BG. Adrenal cortical innervation and glucocorticoid secretion. *J Endocrinol* 1990; 126: 5.
64. Estivariz FE, Morano MI, Carino M, et al. Adrenal regeneration in the rat is mediated by mitogenic N-terminal proopiomelanocortin peptides generated by changes in precursor processing in the anterior pituitary. *J Endocrinol* 1988; 116: 207.
65. Dallman MF, Engeland WC, Rose JC, et al. Nycthemeral rhythm in adrenal responsiveness to ACTH. *Am J Physiol* 1978; 235: R210.
66. Dallman MF, Engeland WC, Shinsako J. Compensatory adrenal growth: a neurally mediated reflex. *Am J Physiol* 1976; 231: 408.
67. Holzwarth MA, Cunningham LA, Kleitman N. The role of adrenal nerves in the regulation of adrenocortical functions. *Ann NY Acad Sci* 1987; 512: 449.
68. Vinson GP, Pudney JA, Whitehouse BJ. The mammalian adrenal circulation and the relationship between adrenal blood flow and steroidogenesis. *J Endocrinol* 1985; 105: 285.
69. Elliot TR. The innervation of the adrenal glands. *J Physiol* 1913; 46: 285.
70. Unsicker K. On the innervation of the rat and pig adrenal cortex. Zeitschrift fur Zellforschung 1971; 116: 151.
71. Robinson PM, Perry RA, Hardy KJ, et al. The innervation of the adrenal cortex in the sheep, Ovis ovis. *J Anat* 1977; 124: 117.
72. Phillips R, Crock C, Funder J. Effects of mineralocorticoids and glucocorticoids on compensatory adrenal growth in rats. *Am J Physiol* 1985; 248: E450.
73. Gagner J-P, Drouin J. Tissue specific regulation of pituitary proopiomelanocortin gene transcription by corticotropin-releasing hormone, 3′,5′-cyclic adenosine monophosphate, and glucocorticoids. *Mol Endocrinol* 1987; 1: 677.
74. Gaspar L, Gilbert F, Chan JSD, et al. Urinary levels of immunoreactive NH2-terminal of pro-opiomelanocortin in patients with malignant pulmonary disease. *Clin Invest Med* 1989; 12: 241.
75. Amara SG, Jonas V, Rosenfeld MG, et al. Alternate RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature* 1982; 298: 240.
76. Nawa H, Kotani H, Nakanishi S. Tissue-specific generation of two preprotachykinin mRNA from one gene by alternate RNA splicing. *Nature* 1984; 312: 729.
77. Phifer JE, Orth DN, Spicer SS. Specific demonstration of the human hypophyseal adrenocortico-melanotropic (ACTH/MSH) cell. *J Clin Endocrinol Metab* 1974; 39: 684.
78. Chrétien M, Seidah NG, Dennis M. Processing of precursor polyproteins in rat brain: regional differences in acetylation of POMC peptides. In: Muller EE, Genazzani AR, eds. *Central and peripheral endorphins: basic and clinical aspects.* New York: Raven Press; 1984: 27.
79. Kakidani H, Furutani Y, Takahashi H, et al. Cloning and sequence analysis of cDNA for porcine β-neo-endorphin/dynorphin precursor. *Nature* 1982; 298: 245.
80. Bohlen P, Wehrenberg WB, Esch F, et al. Rat hypothalamic growth hormone releasing factor: isolation, sequence analysis and total synthesis. *Biochem Biophys Res Comm* 1984; 125: 1005.
81. Lechan RM, Wu P, Jackson IMD, et al. Thyrotropin-releasing hormone precursor: characterization in rat brain. *Science* 1986; 231: 159.
82. Steiner DF, Cunningham D, Spiegelman L, et al. Insulin biosynthesis: evidence for a precursor. *Science* 1967; 157: 697.
83. Rholam M, Nicholas P, Cohen P. Precursors for peptide hormones share common secondary structures forming features at the proteolytic processing sites. *FEBS Lett* 1986; 207: 1.

84. Robbins DC, Blix PM, Rubenstein AH, et al. A human pro-insulin variant at arginine-65. *Nature* 1981; 291: 679.
85. Fletcher DJ, Quigley JP, Bauer GE, et al. Characterization of proinsulin and proglucagon-converting activities in isolated islet secretory granules. *J Cell Biol* 1981; 90: 312.
86. Loh YP, Parish DC, Tuteja R. Purification and characterization of a paired-basic residue-specific pro-opiomelanocortin converting enzyme from bovine pituitary intermediate lobe secretory vesicles. *J Biol Chem* 1985; 260: 7194.
87. Clamagirand C, Camier M, Bousetta H, et al. An endopeptidase associated with ovine neurohypophysis secretory granules cleaves pro-oxytocin neurophysin peptide at paired basic residues. *Biochem Biophys Res Comm* 1986; 134: 1190.
88. Powers CA, Nasjletti A. A novel-kinin-generating protease (kininogenase) in the porcine anterior pituitary. *J Biol Chem* 1982; 257: 5594.
89. Seidah NG, Cromlish J, Hamelin J, et al. Homologous IRCM-serine protease 1 from pituitary, heart atrium and ventricle: a common pro-hormone maturation enzyme? *Biosci Rep* 1986; 6: 835.
90. Julius D, Brake A, Blair L, et al. Isolation of the putative structural gene for the Lys-Arg- cleaving endopeptidase required for processing of yeast prepro-α-factor. *Cell* 1984; 37: 1075.
91. Thomas G, Thorne BA, Thomas L, et al. Yeast KEX2 endopeptidase correctly cleaves a neuroendocrine prohormone in mammalian cells. *Science* 1988; 241: 226.
92. Fuller RS, Brake AJ, Thorner J. Intracellular targeting and structural conservation of a prohormone-processing endoprotease. *Science* 1989; 246: 482.
93. Smeekens SP, Steiner DF. Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease KEX2. *J Biol Chem* 1990; 265: 2997.
94. McKusick VA. The morbid anatomy of the human genome: a review of gene mapping in clinical medicine. *Medicine* 1986; 65: 1.
95. Orci L, Vassali JD, Perrelet A. The insulin factory. *Sci Am* 1988; 259: 85.
96. Chaudhari N, Hahn WE. Genetic expression in the developing brain. *Science* 1983; 220: 924.
97. Van Ness J, Maxwell IH, Hahn WE. Complex population of nonpolyadenylated messenger RNA in mouse brain. *Cell* 1979; 18: 1341.
98. Hökfelt T, Elfvin LG, Elde R, et al. Occurrence of somatostatin like immunoreactivity in some peripheral sympathetic noradrenergic neurons. *Proc Natl Acad Sci USA* 1977; 74: 3587.
99. Hökfelt T, Johansson O, Ljungdahl Å, et al. Peptidergic neurones. *Nature* 1980; 284: 515.
100. Verdoorn TA, Draguhn A, Ymer S, et al. Functional properties of recombinant rat GABA-α receptors depend upon subunit composition. *Neuron* 1990; 4: 919.